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Nicotinamide Benzimidazolide Dinucleotides, Non-Cyclisable Analogues of NAD$^+$

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Abstract: Benzimidazole-based nucleotides and dinucleotides have been synthesised to increase the range of chemical tools available to probe the NAD$^+$ biology space. They were examined for their reactivity in alkylation-type reactions, where they yielded unstable alkylated heteoaromatic adducts, both chemically and enzymatically. While unsuited for NAD$^+$ cyclases, these NAD$^+$ analogues could be viable substrates for non-adenine modifying NAD$^+$-dependent enzyme classes.

Key words: nucleotides, benzimidazoribosyl nucleosides, phosphorylation, cyclic adenosine diphosphoribose, enzymes

There is a need for efficacious methods to access novel analogues of nicotinamide adenine dinucleotide (NAD$^+$) following the recent discoveries of its role in age-related diseases, and therefore probe the vast range of biological events it regulates. For instance, cADPR **1** is an intracellular NAD$^+$ metabolite, generated by intramolecular cyclisation of NAD$^+$ catalysed by an adenosine diphosphate ribosyl transferase (ART).[1] cADPR has now been firmly established as a second messenger, capable of initiating Ca^{2+} release from intracellular stores mediated by the Ryanodine receptor.[2] CD38, the human ART cyclase which also possesses hydrolase activity, is a membrane-bound glycoprotein which has been linked to a range of cellular event regulations including cell activation and muscle contraction to name only a few.[3,4] Importantly, CD38's expression has been linked to poor prognosis in chronic lymphocytic leukaemia.[5]

This cyclase is able to convert a wide range of substrates into cyclic analogues (e.g. nicotinamide N^6-ethenoadenine dinucleotide,[6] a known fluorescent cADPR analogue), which have been used to probe the physiological properties of cADPR and the catalytic properties of this enzyme.[7] Since the discovery of cADPR, both cADPR and NAD$^+$ derivatives such as 3′-F-NAD$^+$[8] have been extensively used in crystal studies of a number of NAD$^+$-converting enzymes,[9] including that responsible for the cyclisation of NAD$^+$ to cADPR. In addition, much of today's knowledge about cADPR-induced Ca^{2+}-signalling pathway was gained due to the wide range of the cADPR analogues synthesised (**2**–**18**).[10–25]

The synthesis of cADPR analogues (**2**–**18**; Figure 1) from novel nucleoside precursors has been achieved using either a total chemical route or a chemoenzymatic approach.[10–25] The chemical route is mostly used to access

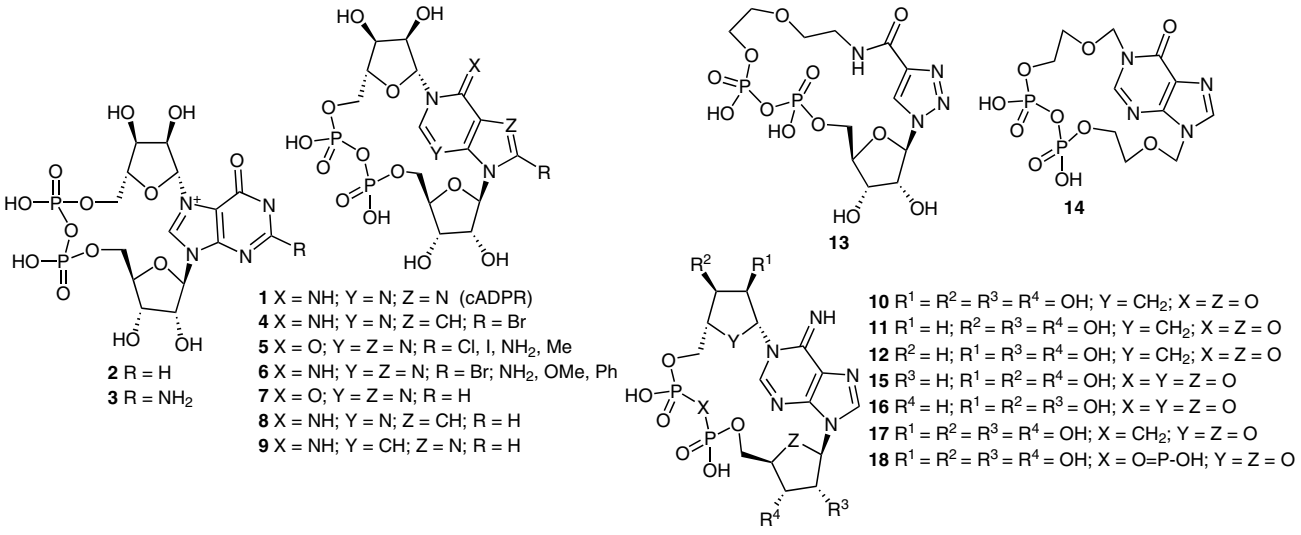

Figure 1 cADPR and analogues successfully synthesised and evaluated for cyclase activity

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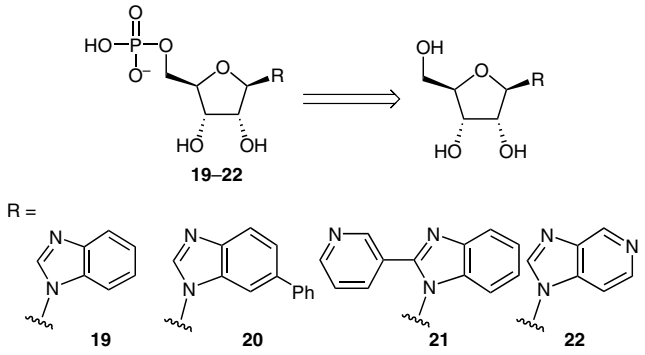
cADPR analogues unattainable through enzymatic cyclisation reactions.[22,26] The chemoenzymatic approach to cADPR derivatives is based on the chemical synthesis of NAD^+ analogues followed by an enzymatic cyclisation using the commercially available adenosine diphosphoribosyl cyclase isolated from *Aplysia californica* (ADPRC). This approach is facilitated by the fact that this latter enzyme has high cyclase activity and low substrate specificity.[27]

While many of these compounds have been designed to investigate the SAR of cADPR for its receptor and its role on calcium release, compounds which inform on the cyclase activity during the cell cycle have been lacking. Therefore, we have been particularly interested in accessing base-modified analogues of NAD^+ that can be alkylated through cyclisation by this class of enzyme and generate analogues which exhibit a change in fluorescence. Till now, neither benzimidazole nor azabenzimidazole NAD^+ and cADPR derivatives have been employed to probe the cyclase activity and calcium release pathway, respectively.[28]

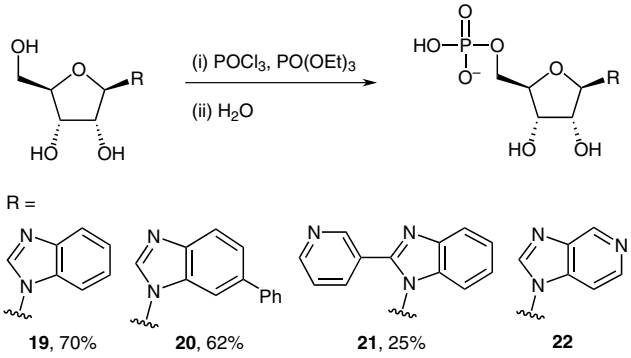
Building on our recently reported three-step one-pot method to synthesise aryl- and heteroaryl-substituted benzimidazoribosyl nucleosides,[29] four of the most readily available nucleoside analogues were selected as precursors in an attempt to generate novel benzimidazole-based cADPR analogues.

It was anticipated that the dinucleotides generated from **19** and **20** would cyclise through the imidazole ring via an N^7–N^9 cyclisation to generate analogues of **2** and **3** while the nucleoside **21** could cyclise via the pyridyl nitrogen and the nucleoside **22** via the fused pyridyl nitrogen. These four nucleosides provide means to access three types of cyclised nucleotides (Scheme 1).



Scheme 1 Nucleotide intermediates **19**–**22**

With these nucleoside analogues at hand,[29] the parameters required for the synthesis of the 5′- phosphate monoesters were to be optimised. Woenckhaus reported the successful preparation of benzimidazole ribonucleotides **19** and **22** while employing a dicyclohexylcarbodiimide coupling reaction in pyridine.[30] This approach required the barium salt form of an appropriately protected phosphate monoester, the 2′,3′-diisopropylidene-protected nucleosides and an extensive deprotection sequence. We therefore aimed to implement a more straightforward phosphorylation protocol using the fully deprotected nucleosides. Consequently, the selective 5′- nucleoside monophosphate was obtained using the Yoshikawa methodology by reaction with $POCl_3$ in $PO(OEt)_3$ with the desired free phosphate achieved through subsequent hydrolysis.[31] For this set of benzimidazole nucleoside derivatives, optimisation of the Yoshikawa reaction method was first conducted on nucleoside **19** and included one molar equivalent of nucleoside dissolved in a minimal volume of triethylphosphate (TEP) by heating,[32] then cooled to 0 °C, followed by the addition of three molar equivalents of phosphorus oxychloride. The reactions were quenched by the addition of ice (Scheme 2).

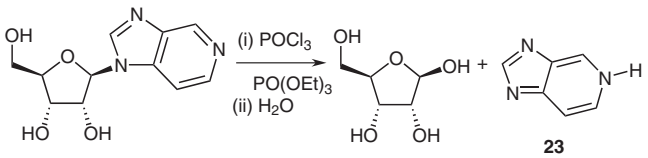


Scheme 2 Phosphorylation of 1-(β-D-ribofuranosyl)benzimidazole and azabenzimidazoles **19**–**22** using phosphorus oxychloride

After overcoming the challenges of TEP removal using chloroform, purification of the nucleotides proved equally difficult. The HPLC purification using ion-exchange chromatography [Q-sepharose Fast Flow or diethylaminoethanol (DEAE) sepharose] proved unsuited as the removal of excess buffered salts could not be accomplished satisfyingly. Nor was the use of C-8-Reversed-Phase (RP) chromatography. HPLC purification of compound **19**[33] was finally achieved in 70% yield using C-18-RP column chromatography with a linear elution gradient of ammonium formate against methanol.

The phosphorylation reaction and the optimised purification procedure were subsequently applied to isolate the pure nucleotides **20**[34] and **21**,[35] in 62% and 25% yield, respectively. Unfortunately, unlike in the sequence reported by Woenckhaus, the nucleoside precursor to nucleotide **22** failed to undergo the Yoshikawa phosphorylation reaction and crude NMR analysis indicated the appearance of quantitative isolation of azabenzimidazole heterocycle **23** (Scheme 3).

Despite the nucleoside precursor to **23** having been synthesised nearly 50 years ago,[30,36] its chemical instability under acidic conditions has not been reported. The Yoshikawa reaction outcome indicates that the azaaryl nitrogen is the most basic and nucleophilic nitrogen of the heteroaromatic moiety, and likely to yield an NAD^+ analogue unsuitable for enzymatic conversion to the corresponding



Scheme 3 Decomposition of azabenzimidazole under Yoshikawa conditions

cADPR analogue, as it is likely to be a chemically unstable species. As a consequence, this class of nucleotide was not pursued.

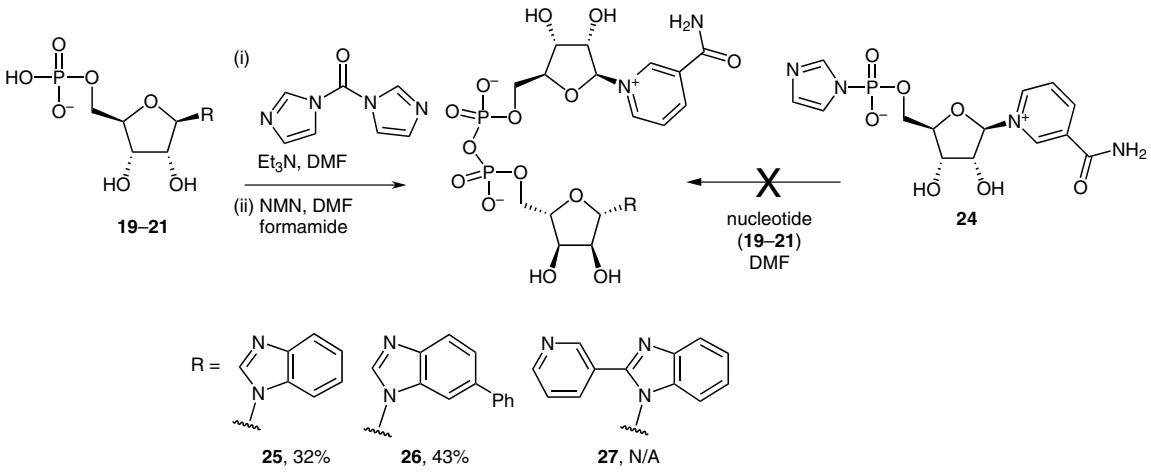
Phosphoroimidazolides have been widely used to access pyrophosphate bonds.[37] These species can be easily prepared via the reaction between a nucleotide and 1,1′-carbonyldiimidazole (CDI) in the presence of a base. The subsequent Mn^{2+}- or Cd^{2+}-catalysed pyrophosphate bond formation led to a range of NAD^+ analogues and nucleotide triphosphates, in neutral aqueous conditions.[15,38,39]

Nicotinamide mononucleotide (NMN), a notoriously unstable molecule towards nucleophiles, was activated to the imidazolidate **24** with CDI in DMF, and the crude was used for its subsequent reaction with **19**–**21**, where the pyrophosphate bond formation could be detected by ^{31}P NMR. Yet, even after extended reaction time, conversion yields to the pyrophosphate remained low. This lack of reactivity is likely related to the low solubility of NMN in DMF, and purification of the crude mixture using a DEAE sepharose anion-exchange column at pH 5 led to the isolation of **25** only in less than 10% yield. Woenckhaus reported similarly low yields, even after extended reaction time for the preparation in pyridine of this dinucleotide under dicyclohexylcarbodiimide conditions.[30]

Consequently, the reaction was repeated changing the order of the reactants and conducted as a two-step one-pot process (Scheme 4). Following the generation of the phosphoroimidazolate derivative of nucleotide **19** using the CDI methodology, addition of β-NMN in a premixed solution of DMF and formamide, yielded the pyrophosphate derivative **25** as detected by ^{31}P NMR (D_2O, br, 2 × P; δ = –10.95, –12.08 ppm).[40] After optimisation of the purification methods, HPLC purification on a DEAE sepharose column with an ammonium formate buffer at pH 5, the dinucleotide **25**[41] was isolated in 32% yield (as measured by UV and Ames assays[42]). The pyrophosphate coupling of the phenyl-substituted benzimidazole nucleotide **20** with β-NMN was performed in the same manner and the NAD^+ analogue **26**[43] was isolated in 43% yield (Scheme 4).

Finally, the coupling reaction between the phosphoroimidazolide derivative of pyridyl-substituted nucleotide **22** (Scheme 2) and NMN proved unsuccessful regardless of the sequence applied. The unsuccessful outcome might be due to the formation of an internal pyridinium phosphate salt which decreases the nucleophilicity of the nucleoside phosphate and decrease its reactivity towards CDI. This statement is supported by the fact that the phosphoimidazolate, intermediate to compound **27**, could not be detected by ^{31}P NMR in the first step of the reaction.

Finally, when attempts were made at establishing the usefulness of these dinucleotides as substrates for the *Aplysia* ART cyclase,[44] no cyclic product could be detected by HPLC–UV (2–24 h incubation time, r.t.; detection at λ = 254 nm; SAX column) even though the NAD^+ derivatives' content declined over time. For the benzimidazolide **25**, the ADPR product generated by hydrolysis of the putative cyclised product would be the same as that generated by simple glycosidic cleavage of the nicotinamide riboside bond. So to investigate whether the decrease in NAD^+ analogues' content over time was due to a lack of reactivity towards the cyclase combined to a lack of stability of the glycosidic nicotinamide bond, or due to the formation of a cyclic ADPR analogue followed by hydrolysis, the nucleoside precursors to **19**–**22** were alkylated in THF–H_2O solution with MeI. The nucleosides rapidly decomposed to the respective methylated nucleobase and ribose derivatives, indicating an unstable nucleosidic C1–'N7' bond being generated upon alkylation.



Scheme 4 CDI methodology for accessing dinucleotide derivatives **25**–**27**

While this chemical behaviour renders these modified NAD^+ compounds inappropriate as cADPR analogue precursors, compound **26** is potentially well suited to differentiate between cyclase and hydrolase activity, since for the former, cyclisation followed by hydrolysis generates ADPR and the isomerised ADPR ('N9' vs 'N7' linkage), while for the latter, only ADPR is generated. Unfortunately under the conditions developed in this work, the breakdown product could only be confirmed by MS as ADPR-like compounds are difficult to detect by HPLC–UV following SAX separation as they are poorly eluted, thus preventing differentiation between the two mechanisms. Yet, this property is potentially useful to examine changes in cyclase vs. hydrolase activity associated with mutations or post-translational modifications, as well as substrate specificity. Work in this area is undergoing to determine the product distribution profiles by methods other than chromatographic separation. However, in view of the chemical space offered by the benzimidazole ring for chemical modifications, a potential application for this class of functionalisable dinucleotides is their use as sirtuins and selective substrates for ADP-ribosyltransferases, where amino group substitution on the benzimidazole would enhance opportunities for sirtuin-specific binding and therefore probing (e.g. as demonstrated for NAD^+ analogues where selective SirT2 vs SirT1 recognition was achieved).[45] This is in addition to the substrate specificity that some of the benzimidazolide dinucleotides have already demonstrated for a range of NAD^+-dependent dehydrogenases.[30]

In conclusion, out of the four benzimidazole riboside derivatives, putative building blocks of cADPR analogues, two benzimidazole NAD^+ analogues have been synthesised effectively, yet in moderate yields. Due to the reduced stability of the protonated or alkylated benzimidazolide nucleotides, none of these building blocks yielded cADPR analogues, however they prove to be potentially useful as reporting probes for cyclase versus hydrolase activity. Additionally, they could potentially be used to probe other NAD^+-dependent processes which include ADP ribosylation of proteins and sirtuin-catalysed deacetylations, an enzymatic event where the nucleobase is not chemically modified.[46]

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(33) **General Procedure for the Yoshikawa Phosphorylation**: Prior to reactions, deprotected nucleosides were dissolved in MilliQ water, solutions frozen and freeze-dried. The powdered nucleoside (100 mg) was dissolved in minimum amount of triethylphosphate and heated with a heatgun until the solution became clear. When the solution cooled to r.t., phosphorus oxychloride was added (3 equiv) and the reaction was left stirring until completion. The reaction's progress was traced by HPLC [SAX anion-exchange column, 50 mM ammonium formate (AF) buffer with 10% MeOH, pH 5]. Once complete, the reaction was quenched by the addition of ice or cold TEAB buffer pH 8. The triethylphosphate was removed by washing the aqueous solution with three portions of ice-cold $CHCl_3$. The crude reaction mixture was then frozen and subsequently freeze-dried, and isolation of the phosphorylated product was performed using a reverse-phase column (Varian: prepacked C18 column); product elution was carried out using a linear gradient 5–90% of 10 mM AF (adjusted to pH 5) against MeOH.
1-*N*-(5′-Phosphato-β-D-ribofuranosyl)benzimidazole (19): A 70% yield of compound **19** was achieved when purification was performed on reverse-phase column (Varian: prepacked C18 column), product elution was carried out using a linear gradient 5–90% of 10 mM AF (adjusted to pH 5) against MeOH. ^{1}H NMR (600 MHz, D_2O): δ = 9.40 (s, 1 H), 7.87–7.93 (m, 1 H), 7.78–7.86 (m, 1 H), 7.58–7.66 (m, 2 H), 6.27 (d, *J* = 4.2 Hz, 1 H), 4.60 (t, *J* = 4.2 Hz, 1 H), 4.39–4.47 (m, 2 H), 4.20–4.27 (m, 1 H), 4.08–4.16 (m, 1 H). ^{13}C NMR (150 MHz, D_2O): δ = 138.3, 131.3, 129.7, 127.2, 126.9, 115.0, 113.2, 90.9, 84.5 (d, *J* = 8.8 Hz), 75.1, 69.7, 63.7 (d, *J* = 4.7 Hz). ^{31}P NMR (162 MHz, D_2O): δ = 0.21. HPLC: t_R = 12.32 min. MS (ES): *m*/*z* [M + H]$^+$ calcd for $C_{12}H_{15}N_2O_7P$: 331.0695; found: 331.0703.

(34) **5-Phenyl-1-*N*-(5′-Phosphato-β-D-ribofuranosyl)-benzimidazole (20)**: A 62% isolated yield of compound **20** was achieved when purification was performed on reverse-phase column (Varian: prepacked C18 column), product elution was carried out using a linear gradient 5–90% of 10 mM AF (adjusted to pH 5) against MeOH. ^{1}H NMR (300 MHz, D_2O): δ = 8.54–8.66 (m, 1 H), 7.97 (m, 1 H), 7.79–7.87 (m, 1 H), 7.53–7.75 (m, 3 H), 7.41 (t, *J* = 7.3 Hz, 2 H), 7.31 (t, *J* = 7.3 Hz, 1 H), 6.08 (d, *J* = 6.2 Hz, 1 H), 4.55–4.62 (m, 1 H), 4.36–4.48 (m, 1 H), 4.22–4.33 (m, 1 H), 4.06–4.16 (m, 2 H). ^{31}P NMR (121 MHz, D_2O): δ = 0.49. HPLC: t_R = 12.88 min. MS (ES): *m*/*z* [M – H]$^-$ calcd for $C_{18}H_{18}N_2O_7P$: 405.0852; found: 405.0869.

(35) **2-(3′-Pyridyl)-1-*N*-(5″-Phosphato-β-D-ribofuranosyl)-benzimidazole (21)**: A 25% yield of compound **21** was achieved when the initial purification was performed on ion-exchange resin (DEAE Sepharose Fast Flow) using gradient miliQ water against 0.1 M triethylamine formate (TEAF) at pH 5 with 10% of MeOH (0–100%) followed by reverse-phase purification (Varian: prepacked C18 column), product elution was carried out using a linear gradient 5–90% of 10 mM AF (adjusted to pH 5) against MeOH. ^{1}H NMR (300 MHz, D_2O): δ = 8.74 (s, 1 H), 8.64 (d, *J* = 4.2 Hz, 1 H), 8.08–8.17 (m, 1 H), 7.96 (d, *J* = 7.6 Hz, 1 H), 7.70 (d, *J* = 7.3 Hz, 1 H), 7.58 (dd, *J* = 5.1, 7.9 Hz, 1 H), 7.30–7.43 (m, 2 H), 5.80 (d, *J* = 7.7 Hz, 1 H), 4.82 (dd, *J* = 6.3, 7.6 Hz, 1 H), 4.35 (dd, *J* = 3.1, 6.3 Hz, 1 H), 4.10–4.16 (m, 1 H), 3.99–4.03 (m, 2 H). ^{13}C NMR (100 MHz, D_2O): δ = 150.5, 149.0, 142.0, 138.7, 132.6, 125.6, 124.6, 124.4, 124.1, 119.1, 113.9, 89.0, 84.2 (d, *J* = 8.7 Hz), 70.7, 69.6, 64.1 (d, *J* = 4.8 Hz). ^{31}P NMR (121 MHz, D_2O): δ = 2.24. HPLC: t_R = 15.38 min. MS (ES): *m*/*z* [M + H]$^+$ calcd for $C_{17}H_{19}N_3O_7P$: 408.0961; found: 408.0952.

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(40) **General Procedure for the CDI-Mediated Pyrophosphate Bond Formation**: Prior to the reaction a stock solution of NMN was prepared by dissolving NMN (100 mg) in anhyd DMF (1 ml) and anhyd formamide (1 mL). CDI (2.5 equiv) and Et_3N (2 equiv) were then added to a solution of nucleotide (1.2 equiv) in anhyd DMF (0.5 mL). The reaction was stirred for 12 h at r.t. The reaction was then quenched with anhyd MeOH and evaporated to dryness. The resulting solid was dissolved in new portion of anhyd DMF (0.5 mL) and a solution of NMN (1 equiv) was added. The reaction was followed by ^{31}P NMR and once the imidazolate peak had disappeared, the reaction was diluted with MilliQ water and applied on DEAE sepharose. Isolation of the NAD analogues was performed using ion-exchange resin (DEAE Sepharose Fast Flow) using gradient MilliQ water against 0.1 M ammonium formate buffer (AF) at pH 5 with 10% of MeOH (0–100%).

(41) **Nicotinamide Benzimidazole Dinucleotide (25)**: The product was isolated after 48 h in 32% of yield. ^{1}H NMR (400 MHz, D_2O): δ = 9.12 (s, 1 H), 8.98 (d, *J* = 6.2 Hz, 1 H), 8.61 (d, *J* = 8.1 Hz, 1 H), 8.38 (s, 1 H), 7.94–8.07 (m, 1 H), 7.58 (d, *J* = 7.58, 15.6 Hz, 2 H), 7.25–7.34 (m, 3 H), 6.98 (d, *J* = 7.8 Hz, 1 H), 5.91 (dd, *J* = 5.9, 9.3 Hz, 2 H), 3.98–4.51 (m, 10 H). ^{13}C NMR (100 MHz, D_2O): δ = 180.0, 171.0, 145.4, 142.1, 142.1, 139.7, 133.4, 129.9, 128.5, 124.2, 123.5, 121.6, 119.1, 114.9, 111.5, 99.9, 88.2 (d), 83.7 (d), 77.5, 72.9, 70.4, 70.10, 65.59 (d), 64.81 (d). ^{31}P NMR (162 MHz, D_2O): δ = –10.95 to –12.08 (m, P–O–P). MS (ES): *m*/*z* [M – H]$^-$ calcd for $C_{23}H_{28}N_4O_{14}P_2$: 646.1077; found: 646.1078. HPLC: t_R = 7.17 min.

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(43) **Nicotinamide 5-Phenylbenzimidazole Dinucleotide (26)**: The product was isolated after 48 h in 43% of yield. ^{1}H NMR (400 MHz, D_2O): δ = 8.86 (s, 1 H), 8.67 (s, 1 H), 8.58 (s, 2 H), 8.09 (s, 1 H), 7.85 (s, 1 H), 7.29–7.68 (m, 9 H), 5.91 (s, 1 H), 5.52 (s, 1 H), 3.94–4.42 (m, 10 H). ^{31}P NMR (162 MHz, D_2O): δ = –11.09 to –11.60 (m, P–O–P). HPLC: t_R = 8.87 min. MS (ES): *m*/*z* [M]$^+$ calcd for $C_{29}H_{33}N_4O_{14}P_2^+$: 723.1469; found: 723.1436.

(44) *Aplysia* cyclase was purchased from Sigma-Aldrich. The incubation reactions were monitored by HPLC (SAX column, 5% MeOH; 50 mM KH_2PO_4, pH 4). The enzymatic

assays were conducted in TEAB buffer (0.1 M, pH 7.2) and used 1 mL solution containing 100 mM of NAD^+ analogues, and 10 μL of reconstituted enzyme in buffer (ca. 300 units/mL).

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